Example 18: Methods of Synthesizing Oligonucleotide-Nanoparticle Conjugates And The Conjugates Produced By The Methods

A. General Methods

$HAuCl_4 \cdot 3H_2O$ and trisodium citrate were purchased from Aldrich chemical company, Milwaukee, WI. Gold wire, 99.999% pure, and titanium wire were purchased from Goldsmith Inc., Evanston, IL. Silicon wafers (100) with a 1 micron thick oxide layer were purchased from Silicon Quest International, Santa Clara, CA. 5'-thiol-modifier C6-phosphoramidite reagent, 3'-propylthiol modifier CPG, fluorescein phosphoramidite, and other reagents required for oligonucleotide synthesis were purchased from Glen Research, Sterling, VA. All oligonucleotides were prepared using an automated DNA synthesizer (Expedite) using standard phosphoramidite chemistry (Eckstein, F. *Oligonucleotides and Analogues*; 1st ed.; Oxford University Press, New York, 1991). Oligonucleotides containing only 5' hexylthiol modifications were prepared as described in Example 1. 5-(and 6)-carboxyfluorescein, succinimidyl ester was purchased from Molecular Probes, Eugene, OR. NAP-5 columns (Sephadex G-25 Medium, DNA grade) were purchased from Pharmacia Biotech. Nanopure H_2O (>18.0 MΩ), purified using a Barnstead NANOpure ultrapure water system, was used for all experiments. An Eppendorf 5415C or a Beckman Avanti 30 centrifuge was used for centrifugation of Au nanoparticle solutions. High Performance Liquid Chromatography (HPLC) was performed using a HP series 1100 HPLC.

B. Physical Measurements.

Electronic absorption spectra of the oligonucleotide and nanoparticle solutions were recorded using a Hewlett-Packard (HP) 8452a diode array spectrophotometer. Fluorescence spectroscopy was performed using a Perkin-Elmer LS50 fluorimeter. Transmission Electron Microscopy (TEM) was performed with a Hitachi 8100 Transmission Electron Microscope operating at 200 kV. A Thermo Jarrell Ash AtomScan 25 atomic emission spectrometer with an inductively coupled plasma (ICP) source was used to determine the atomic concentration of gold in the nanoparticle solutions (gold emission was monitored at 242.795 nm).

C. Synthesis and Purification of Fluorescein-Labeled Alkanethiol-Modified Oligonucleotides

Thiol-modified oligonucleotide strands containing either 12 or 32 bases, with 5' hexylthiol and 3' fluorescein moieties, were prepared. The sequence of the 12mer (S12F) was $HS(CH_2)_6$-5'-CGC-ATT-CAG-GAT-3'-$(CH_2)_6$-F [SEQ ID NO:50], and the 32mer (SA_{20}12F) contained the same 12mer sequence with the addition of a 20 dA spacer sequence to the 5' end [SEQ ID NO:51]. The thiol-modified oligonucleotides were prepared as described in Storhoff et al., *J. Am. Chem.Soc.* **120**:1959-1964 (1998). An amino-modifier C7 CPG solid support was used in automated synthesis, and the 5' terminus was manually modified with hexylthiol phosphoramidite, as described previously. The 3' amino, 5' trityl-protected thiol modified oligonucleotides were purified by reverse-phase HPLC using an HP ODS Hypersil column (5 mm, 250 x 4 mm) with 0.03 M triethyl ammonium acetate (TEAA), pH 7 and a 1% / minute gradient of 95% CH_3CN / 5% 0.03 M TEAA at a flow rate of 1 mL/min., while monitoring the UV signal of DNA at 254 nm. The retention times of the 5'-S-trityl, 3' amino modified 12-base and 32-base oligonucleotides were 36 and 32 minutes respectively.

The lyophilized product was redispersed in 1 ml of 0.1 M Na_2CO_3 and, while stirring in the dark, 100 µL of 10 mg/ml succinimidyl ester of fluorescein (5,6 FAM-SE, Molecular Probes) in dry DMF was added over 1.5 hours according to the directions of the manufacturer (Molecular Probes literature). The solution was stirred at room temperature for an additional 15 hours, then precipitated from 100% ethanol at -20 °C. The precipitate was collected by centrifugation, dissolved in H_2O and the coupled product separated from unreacted amino-terminated oligonucleotide by ion-exchange HPLC. A Dionex Nucleopac PA-100 column (250 x 4 mm) was operated with 10 mM NaOH aqueous eluent and a 1% / minute gradient of 1 M NaCl/10mM NaOH at a flow rate of 0.8 mL/minute. Retention times of 5'-S-trityl, 3' fluorescein modified 12mer and 32mer were 50 and 49 minutes respectively. The oligonucleotide product was desalted by reverse-phase HPLC. Removal of the trityl protection group of the fluorescein-terminated, trityloligonucleotide was

performed using silver nitrate and dithiothreitol (DTT) as previously described (Storhoff et al., *J. Am. Chem.Soc.* **120**:1959-1964 (1998)). The yield and purity of the oligonucleotides were assessed using the techniques previously described for alkylthiol oligonucleotides (Storhoff et al., *J. Am. Chem.Soc.* **120**:1959-1964 (1998)). Oligonucleotides were used immediately after detritylation of the thiol group.

Thiol-modified oligonucleotides containing 32 bases, with 3' propylthiol and 5' fluorescein moieties ($HS(CH_2)_3$-3'-$(W)_{20}$-TAG-GAC-TTA-CGC-5'-$(CH_2)_6$-F, W= A or T) [SEQ ID NO:52] were synthesized on an automated synthesizer using 3' thiol modifier CPG. The 5' terminus of each oligonucleotide was coupled manually to fluorescein phosphoramidite (6-FAM, Glen Research). The modified oligonucleotides were purified by ion exchange HPLC (1% / min gradient of 1 M NaCl, 10 mM NaOH; retention time (Rt) ~ 48 min (W = T), Rt ~ 29 min (W = A)). After purification, the oligonucleotide solutions were desalted by reverse phase HPLC. The 3' thiol moieties were deprotected with dithiothreitol by a procedure previously described (Storhoff et al., *J. Am. Chem.Soc.* **120**:1959-1964 (1998)).

D. Synthesis and Purification of Fluorescein Labeled Oligonucleotides.

The fluorophore labeled complement (12'F) consisted of 12 bases 3'–GCG-TAA-GTC-CTA–5'-$(CH_2)_6$-F [SEQ ID NO:53] complementary to the 12mer sequence in S12F and SA_{20}12F. The oligonucleotide was synthesized using standard methods, and a syringe-based procedure, similar to the procedure reported above for the 5' alkylthiol modification, was used to couple fluorescein phosphoramidite (6-FAM, Glen Research) to the 5' end of the CPG-bound oligonucleotide. Purification was performed using reverse-phase HPLC as above. The fluorescein-labeled oligonucleotide had a retention time of 18 min. The fluorophore labeled complement, 3'12F (5'-ATC-CTG-AAT-GCG-F; [SEQ ID NO:54]) was prepared using an amino-modifier C7 CPG solid support for automated synthesis, followed by coupling of 5-(6)-carboxyfluorescein succinimidyl ester to the 3' amine using the procedure described above.